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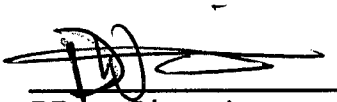

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ABSTRACT

The NF-κB/Rel family of dimeric transcription factors has been shown to promote cell survival, and increasing evidence suggests involvement in carcinogenesis. Recently, NF-κB/Rel was found to be constitutively active in the nuclei of human breast cancer cell lines, as well as in 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors from Sprague-Dawley rats (S-D). Malignantly transformed human mammary epithelial cells (HMEC), derived by carcinogen treatment of non-tumorigenic parental MCF-10F cells displayed increased constitutive NF-κB activation. In premalignant HMECs immortalized by carcinogen treatment *in vitro,* NF-κB activity was dysregulated in quiescence. Six founder lines of transgenic mice with targeted ectopic expression of the c-Rel subunit in the mammary gland were established, and studies are in progress to directly test the role of NF-κB/Rel in the mammary gland. AhR and RelA synergized to transactivate the c-*myc* promoter in the MCF-10F HMECs, as well as in the Hs578T breast cancer cells. These results suggest that the activation of NF-κB/Rel and AhR may be critically involved in proliferation and/or malignant transformation of the mammary gland and its functional target may be the c-*myc* proto-oncogene. Overall, these studies provide evidence for involvment of the AhR, NF-κB/Rel, and c-Myc proteins in a common pathway towards malignant progression of mammary epithelial cells.

I. Introduction

NF-κB/Rel is a family of transcription factors, which are expressed in all cells; however, in most non-B cells, they are sequestered in the cytoplasm in inactive complexes with specific inhibitory proteins, termed IκBs. We have recently shown that NF-κB/Rel factors are aberrantly activated in breast cancer, and function to promote tumor cell survival. Specifically, mammary tumors induced upon carcinogen treatment of Sprague-Dawley (S-D) rats, human breast tumor cell lines, and primary human breast tumor tissue samples were found to constitutively express high levels of nuclear NF-κB/Rel, whereas normal rat mammary glands and untransformed breast epithelial cells contained the expected low basal levels. Inhibition of this activity in breast cancer cells in culture via introduction of the specific inhibitory protein IκB-α led to apoptosis. Furthermore a time course study of induction of NF-κB/Rel factors upon carcinogen treatment of female S-D rats revealed that NF-κB/Rel activation was an early event, occurring prior to malignant transformation. In the first year of the research fellowship I have pursued the following objectives:

1. Quantitate and characterize the NF-κB/Rel subunits induced in the 7,12-dimethylbenz(*a*)anthracene (DMBA) and benzo[*a*]pyrene (BaP) transformed human mammary epithelial cell (HMEC) lines D3-1 and BP-1.
2. Determine the kinetics of NF-κB/Rel induction in the carcinogen transformation process by studying the activation/function of NF-κB/Rel in the premalignant BaP transformed 184A1 cells.
3. Establishment and characterization of MMTV-cRel transgenic mice.
4. Analysis of the interaction of aryl hydrocarbon receptor transcription factor (AhR) and NF-κB/Rel proteins in the human mammary epithelial cells.

II. Body

Projects in Progress

Project 1. Carcinogen Transformed Cell Line Analysis:

A. Malignant Lines Analysis:

MCF-10F cell line was established from mammary tissue from a patient with fibrocystic disease. As a collaboration with Dr. Russo, we have obtained the 7,12 dimethylbenz(a)anthracene (DMBA), and benzo[a]pyrene (BaP) transformed derivatives of this cell termed D3-1 and BP-1, respectively. BP-1 cells exhibit increased anchorage independent growth, increased chemotaxis and invasiveness. D3-1 cells exhibit similar traits but to a lesser extent than that of BP-1.

EMSA analysis revealed that D3-1 and BP-1 cells exhibit stronger binding of NF-κB oligonucleotide probe than the parental MCF-10F cells (Fig. 1A). Supershift analysis revealed that p65/p50 and p50/p50 dimers are present in the binding complexes of the D3-1 (Fig. 1B) similar to those found in the MCF-10F cells. Functionality of the NF–κB activity was assessed by performing transient transfection analysis (Fig. 1A). Wildtype (E8) and mutant (mutE8) NF-κB element-thymidine kinase (TK) promoter-chloramphenicol acetyltransferase (CAT) reporter vectors were used for the analysis. Briefly, these consisted of 2 copies of either the wild type NF-κB element from upstream of the c-*myc* promoter, or versions with the two internal G residues converted to C residues, which significantly reduces NF-κB binding and transactivation. MCF-10F cells displayed 1.6 to 2.6-fold wildtype E8 over mutant E8 activity. D3-1 cells displayed 4.1-fold higher levels of wildtype E8 activity over the mutant. Specificity of this activity was assessed by co-transfection with an IκB-α expression plasmid, and downregulation of the activity was noted (data not shown).

Similar analysis was also performed on the BP-1 cells. These cells also exhibited stronger binding of NF-κB oligonucleotide probe than the parental MCF-10F cells (Fig. 1A). Supershift analysis revealed that p65/p50 and p50/p50 dimers in the binding complexes (data not shown). Functionality of the NF-kB activity was again assessed by transient transfection analysis. The BP-1 cells displayed 11.6-fold higher E8 over the mutant activity (Fig. 1A). Specificity of this activity was again demonstrated by co-transfection with an IκB-α expression plasmid (data not shown).

Since the rate of IκB-α turnover controls NF-κB release, protein turnover assays were performed in these cells to determine if a decrease in IκB-α stability was responsible for this increased NF-κB activity. The results clearly indicate that the turnover rate of IκB-α is markedly increased in the D3-1 and BP-1 cells compared to its parental 10F cells (Fig. 2). Thus, it appears that a potential mechanism of NF-κB activation in these transformed cells may be due to dysregulation of the IκB-α stability.

B. Pre-malignant Lines Analysis

Mika Sovak, a former graduate student in the lab, and Greg Zanieski, a former technician, demonstrated that NF-κB/Rel binding is activated in the mammary glands of DMBA-administered rats prior to tumor formation. Thus, we hypothesized that the NF-κB activation might be an early event in the transformation process. Dr. Stampfer has recently established an

orderly staging of progression of human mammary epithelial cells (HMEC) from a mortal to an immortalized yet nonmalignant phenotype following BaP treatment. 184 HMEC lines were established from a reduction mammoplasty tissue and senesce after 22 passages. Extended lifespan 184Aa clone was isolated post in vitro treatment of 184 cells with BaP. Indefinite lifespan 184A1 cells appeared from 184Aa at p9. We initiated collaboration with Dr. Stampfer, and obtained nuclear extracts of fine lifespan 184 HMECs, and late passage (fully immortal) 184A1 HMECs. EMSA demonstrates that there is very little κB binding activity for the finite lifespan 184 HMECs at both quiescence (G0) and cycling stages. However, the fully immortal premalignant 184A1 cells at quiescence displayed a very high binding activity similar to that of the Hs578T human breast tumor cells (Fig. 3). The increased NF-κB binding of 184A1 cells only when G0 arrested, suggests a dysregulation of NF-κB activation in these cells at quiescence. Transient transfection analysis of E8 kappaB reporter construct confirmed this dysregulation in the fully immortal 184A1 cells. Supershift analysis revealed that p65 and p50 subunits are the primary binding subunits of NF-κB in both the finite lifespan 184 and the fully immortal 184A1 cells (data not shown). Thus, these results reveal that NF-κB activation is dysregulated in the premalignant cells in quiescence.

Project 2. MMTV c-Rel Transgenic Mice

A. Founder Copy Numbers and phenotypes:

Six lines of transgenic mice (lines 7,8,14,15, 16, & 18) have been established by the Core Transgenic Facility of Boston University Medical Center, and confirmed by Southern blot analysis. Each mice exhibits varying copy numbers of MMTV-c-Rel DNA.

Founder Number	Approx. Copy Number	Date of Birth
7	3.7	12/28/97
8	6.22	12/28/97
14	4.74	3/16/98
15	4.5	3/16/98
16	8.75	3/16/98
18	2.58	3/16/98

1. Founder 7 – We found an interesting trait for founder 7's progenies. The male mice are unable to produce more than 5 pups in a litter. Average pup size appears to be about 4. (4,5,3,5,5,5,3,2,4,4,5). These mice have been bred to F2's and potential homozygotes are being tested.
2. Founders 8, 14 – No remarkable phenotype as of yet. They've also been bred down to F2's and potential homozygotes are being tested.
3. Founder 15 – Initially appeared to have small pup size, however, now appears to be breeding fine. A pregnant mouse from founder 15 developed a cystic lesion extending from the L1/2 mammary gland.
4. Founder 16 – Is the line with the highest copy number. Initially, there was noticeably a higher number of male to female ratio, but now appears to no longer have this discrepancy. A virgin mouse from founder 16 developed an ovarian cystic teratoma.

B. Analysis:

Wholemount was performed from a transgenic mouse from each of the lines. No apparent morphological abnormalities were detected upon analysis. Preliminary immunoblot and EMSA studies indicate enhanced c-Rel nuclear levels, but not binding to an NF-κB oligonucleotide. Hence, in future studies, experiments are planned to test whether they will display enhanced tumor formation upon carcinogen treatment.

Project 3: Ahr and NF-κB cooperate to induce the c-*myc* promoter.

Recent studies have shown that p65 subunit and the aromatic hydrocarbon receptor/transcription factor (AhR) co-immunoprecipitate, and in some cases inhibit promoters driven by multimerized NF-κB elements. Dr. Sherr's lab (Boston University School of Public Health, Boston MA) has demonstrated that the p65 subunit co-immunoprecipitates with AhR in extracts from the MCF-10F cells and Hs578T cells, and that AhR seems to be involved in proliferation of these cells. Since our lab had previously demonstrated that the c-*myc* proto-oncogene has two NF-κB elements, we investigated the potential role of AhR and NF-κB in cooperating to induce the c-*myc* gene, in collaboration with Dr. Sherr's lab. I have performed transient transfection analysis and demonstrated that in the MCF-10F cells (Fig. 4) and Hs578T cells (data not shown), the p65 subunit and AhR can activate the c- *myc* promoter cooperatively. A c-*myc* promoter construct that has mutations in the URE and IRE (NF-κB elements), failed to be transactivated by the AhR and p65 (Fig. 4). RelB, and c-Rel subunits failed to cooperate with the AhR in transactivating the c- *myc* promoter (data not shown). Furthermore, EMSA analysis using the URE NF-κB oligonucleotide probe revealed that transfection of AhR and p65 subunit leads to an induction of a band that migrates more slowly than the classical p65/p50 subunit (Fig. 5A). This band is specific, as judged by cold oligonucleotide competition analysis (Fig 5B), and can be block shifted by both the p65 and AhR antibody suggesting that AhR and p65 subunits are in the binding complex (Fig. 5C and D). Thus, these preliminary findings suggest an exciting new role of AhR and p65 function.

III. Figures:

Fig. 1. Carcinogen-transformed D3-1 and BP-1 cells display higher constitutive levels of functional NF-κB than the parental MCF-10F cells. **A)** Comparison of lines. The MCF-10F cells (10F) and BP-1 cells were transiently transfected by lipofection, in triplicate or duplicate, respectively with 2 ug E8 or mutE8 reporter construct. Alternatively, D3-1 cells were transfected, in duplicate, using 20ug of either E8 or mutE8 by the calcium phosphate method. After 24 hours (for lipofectamine) or 72 hours (for calcium phosphate), extracts were prepared, normalized for protein, and assayed for CAT activity. The values for E8 CAT activity are represented as fold induction over mutE8 CAT activity which was set at 1.0 for each cell line. Shown is the representative data from a minimum of 2 experiments. (Inset) Equal amounts (5 ug) of nuclear extracts from exponentially growing parental MCF-10F cells or transformed D3-1 or BP-1 cells were subjected to EMSA with a radiolabeled oligonucleotide NF-κB element as probe. A representative experiment of two independent assays is shown.
B) Supershift analysis of D3-1 cells. Following a 30 min incubation of nuclear extracts (5 ug) with the probe, 1 ul of antibody against the p50 (sc-114), p65 (#1226, kindly provided by N. Rice) or c-Rel (sc-070) was added as indicated, incubated and subjected to EMSA.

Fig. 2. IκB-α protein in D3-1 and BP-1 cells has a shorter half life than in parental MCF-10F cells. **A)** MCF-10F, D3-1 and BP-1 cells were incubated in the absence or presence of 20 ug/ml emetine for the indicated periods of time. Cytoplasmic extracts (50 ug protein/lane) were separated by electrophoresis in a 10% polyacrylamide-SDS gel, and subjected to immunoblot analysis for IκB-α protein using SC-371 antibody. These blots are representative of two experiments. **B)** The immunoblot for IκB-α protein in part A was quantitated by densitometry, and the data plotted as percent of the original protein value in untreated control cells (0 hr). The decay curves were extrapolated by using an exponential best fit analysis.

Fig. 3. Dysregulated NF-κB/Rel expression in 184A1 immortalized HMECs. **A)** 184A1 cells display increased NF-κB binding in quiescence. EMSA was performed with nuclear extracts (5 ug) from finite lifespan 184 and fully immortal 184A1 cells following G_0 synchronization upon blockage of EGF receptor signal transduction for 48 hours (G0), or during exponential growth (CYC). Two distinct NF-κB binding complexes were detected.

Fig. 4. RelA and AhR cooperate to transactivate the wildtype p1.6Bgl, but not the p1.6Bgl dbl mut, c-*myc* promoter construct. Confluent MCF-10F cells (~ 200,000 cells in 35 mm^2 dishes) were transiently transfected, in duplicate, with either 1 μg p1.6 Bgl or p1.6 Bgl dbl mut, and 0, 2, or 4 μg *pcDNA3-AhR* (murine AhR) expression vector in the absence or presence of 0.125 μg *pEVRF-p65* (RelA expression) plasmid using 7 μl FUGENE reagent. In each transfection, 1 μg of TK-luciferase plasmid was added as an internal control for normalization of transfection efficiency. Total DNA transfected was maintained at 6 μg by addition *pcDNA3* plasmid (parent vector for *pcDNA3-AhR*). Transfected cells were harvested after 24 h in reporter lysis buffer, and analyzed for CAT and luciferase activity. CAT activities are presented normalized for transfection efficiency, using the luciferase activity.

Fig. 5. Expression of RelA and AhR yields a novel URE NF-κB element binding complex. **A)** Co-transfection with AhR and RelA expression vectors leads to formation of a novel complex. Confluent plates (100 mm^2 dishes) of Hs578T cells were transfected with either 52 μg *pcDNA3* empty vector, or 50 μg *pcDNA3-AhR* in the absence or presence of 2 μg *pEVRF-p65* expression plasmid using 70 μl FUGENE reagent. After 24 h, nuclear proteins were isolated using the method of Dignam et al. (22), and subjected to EMSA. N indicates position of a new complex; 1, indicates position of previously observed major complex. **B)** Competition EMSA confirms the specificity of the major bands. Nuclear extracts of Hs578T cells co-transfected with *pcDNA3-AhR* and *pEVRF-p65* were pre-incubated with either 4- or 20-fold molar excess unlabelled wildtype (URE) or mutant (mt URE) URE prior to the 30 min incubation reaction with the radiolabelled URE. Two nonspecific bands were identified and marked with an asterix (*). **C)** Nuclear extracts from the AhR and RelA expression vector co-transfected cells, prepared as described above in Fig. 3, were incubated with the URE probe. Following a 30 min binding reaction, antibodies were added where indicated, the reactions incubated for an additional 1 h, and subjected to EMSA. Results of two separate analyses are shown. **D)** lane 1, no antibody; lane 2, 1 μl RelA-specific antibody (#1226, kindly provided by N. Rice), lane 3, 1 μl AhR-specific antibody (BioMol #SA-210); Two nonspecific bands were identified and marked with an asterix (*). A faster migrating, nonspecific band, that appears upon addition of antibody #1226 with the probe alone, is indicated by a double asterisk (**). B) lane 1, no antibody; 1 μl RelA-specific antibody (sc-372X); lane 3, 1 μl p50-specific antibody (sc-114). Specific binding complexes are indicated as band 1 and band N, as above.

Appendix

1. List of key research accomplishments:
   - NF-κB is functionally activated in HMECs malignantly transformed by environmental carcinogens
   - In premalignant HMECs immortalized by carcinogen treatment *in vitro,* NF-κB activity was dysregulated in quiescence.
   - Six founder lines of transgenic mice with targeted ectopic expression of the c-Rel subunit in the mammary gland were established, and studies are in progress to directly test the role of NF-κB/Rel in the mammary gland
   - Aromatic Hydrocarbon Receptor (AhR) and RelA (p65) cooperate to transactivate the c-*myc* promoter in the MCF-10F HMECs, as well as in the Hs578T breast cancer cells
2. Degrees Obtained
   - Ph.D. defense passed by the Principal Investigator. Formal degree to be awarded on June of 2001 upon completion of M.D. degree at Boston University School of Medicine.
3. Manuscripts/Presentations

   - D. W. Kim, L. Gazourian, S. A. Quadri, R. Romieu, D. H. Sherr, and G. E. Sonenshein. The Aromatic Hydrocarbon Receptor/Transcription Factor (AhR) and the p65 Nuclear Factor-κB Subunit Cooperate to Transactivate the c-*myc* Promoter. Manuscript submitted to The Journal of Biological Chemistry, 1999.

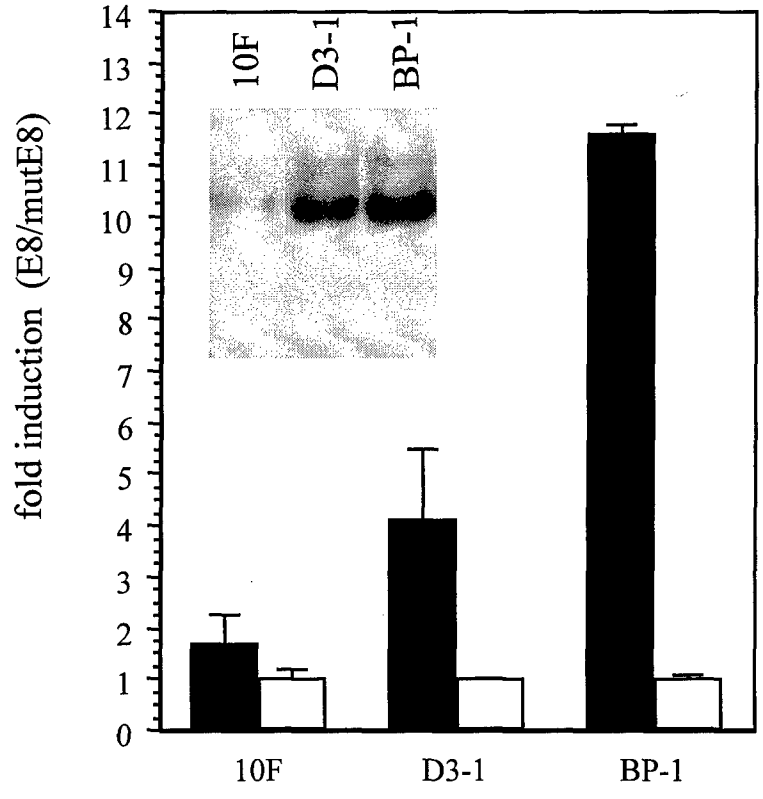
   - D.W. Kim, M.A. Sovak, G. J. Zanieski, G. Nonet, P. Yaswen, M. Stampfer, J. Russo, A.E. Rogers, G.E. Sonenshein. Activation of NF-κB/Rel Occurs Early During Neoplastic Transformation of Mammary Cell. Manuscript submitted to Carcinogenesis. 1999.

   - D.W. Kim, M.A. Sovak, M. Arsura, G. J. Zanieski, K. Kavanagh, G. Nonet, P. Yaswen, M. Stampfer, J. Russo, A.E. Rogers, G.E. Sonenshein. Early Activation of NF-κB/Rel During Neoplastic Transformation of Mammary Cells. Russek Day Presentation, Boston Unviersity School of Medicine (2nd Prize Award).

(Copies of the manuscripts will be furnished upon receipt of reprints when manuscripts are accepted for publication)

Figure 1

A)



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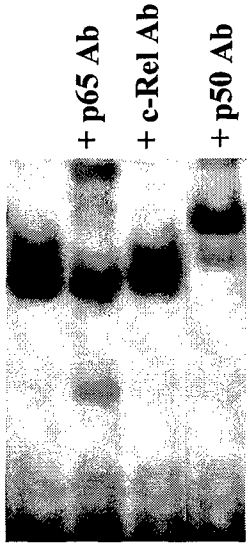

D3-1

Figure 2

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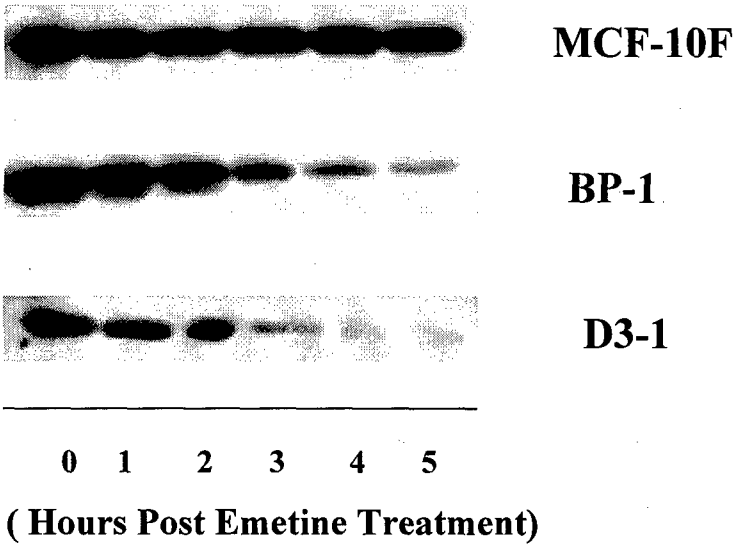
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BP-1
D3-1
0 1 2 3 4 5
(Hours Post Emetine Treatment)

B)

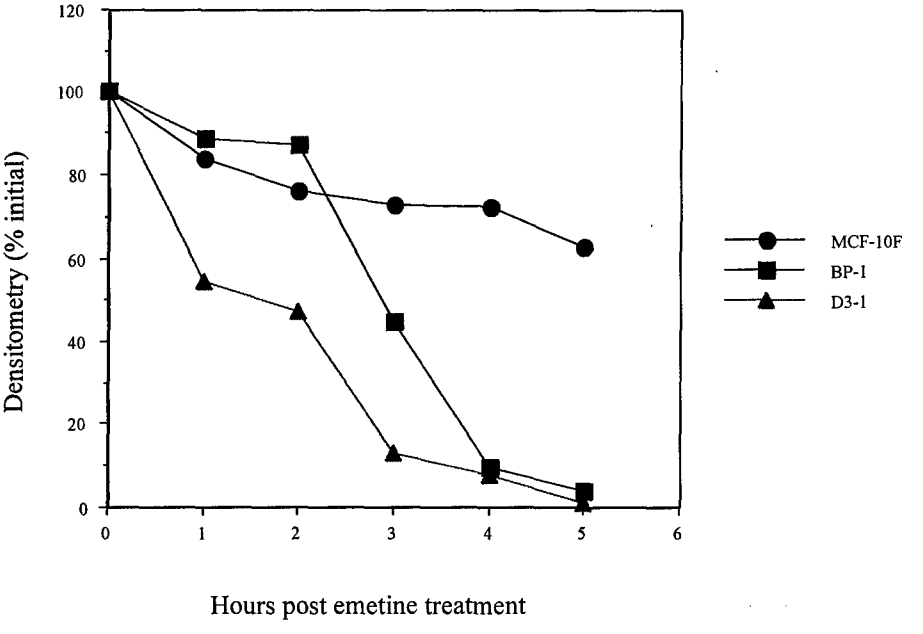
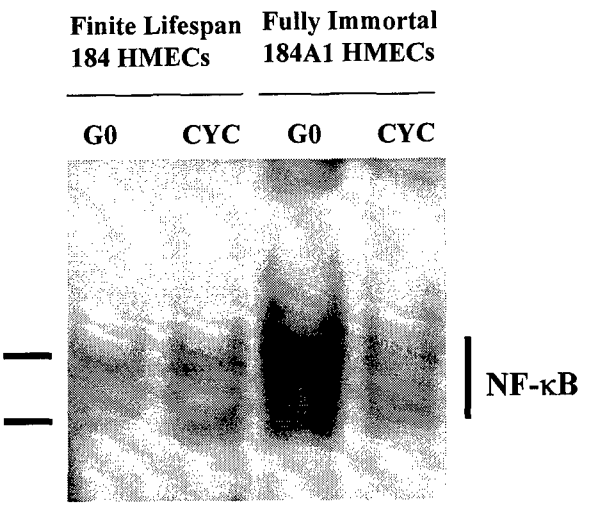

Densitometry (% initial)
120
100
80
60
40
20
0
0 1 2 3 4 5 6
Hours post emetine treatment
MCF-10F
BP-1
D3-1

Figure 3



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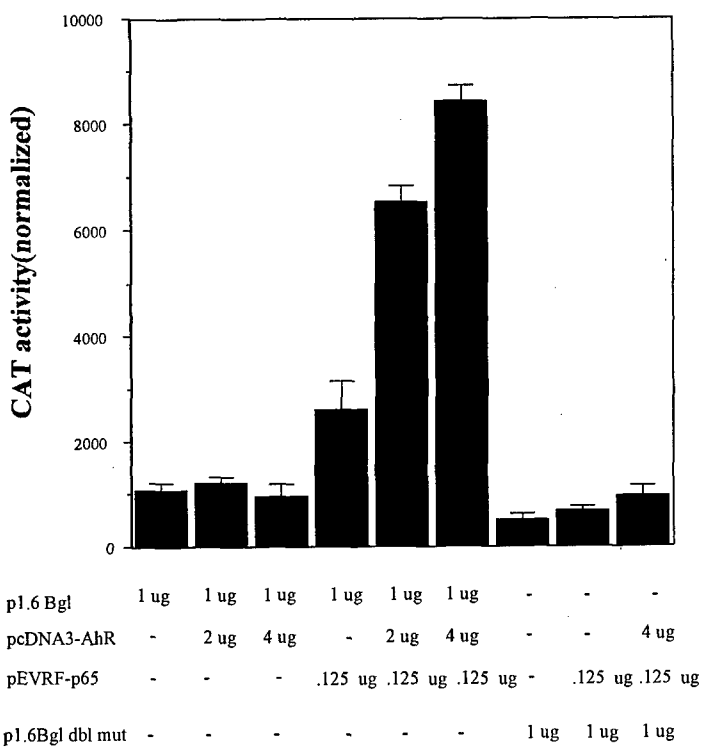
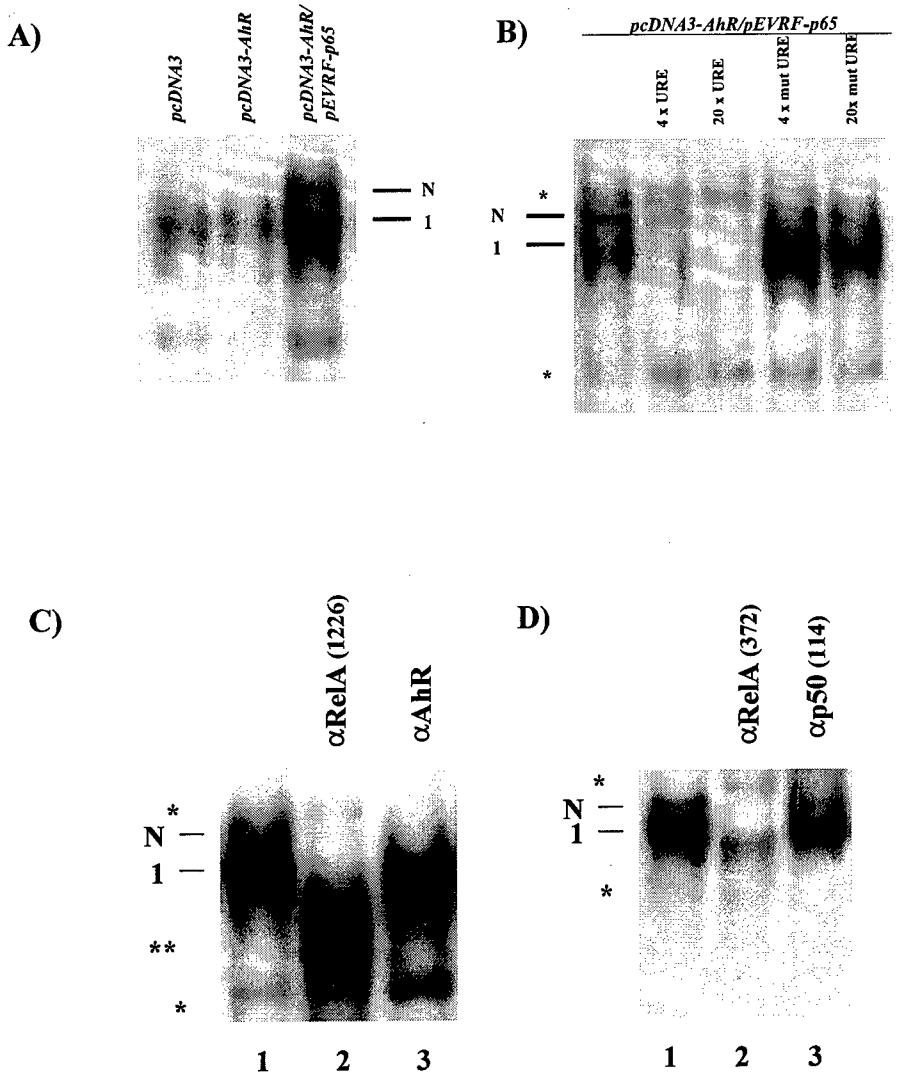


Figure 5


A)
pcDNA3
pcDNA3-AhR
pcDNA3-AhR/ pEVRF-p65
N
1
B)
pcDNA3-AhR/pEVRF-p65
4 x URE
20 x URE
4 x mut URE
20x mut URE
N
1
*
*
C)
αRelA (1226)
αAhR
N
1
*
**
*
1
2
3
D)
αRelA (372)
αp50 (114)
N
1
*
*
1
2
3